A Nanobody-Based System Using Fluorescent Proteins as Scaffolds for Cell-Specific Gene Manipulation

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SUMMARY

Fluorescent proteins are commonly used to label cells across organisms, but the unmodified forms cannot control biological activities. Using GFP-binding proteins derived from Camelid antibodies, we co-opted GFP as a scaffold for inducing formation of biologically active complexes, developing a library of hybrid transcription factors that control gene expression only in the presence of GFP or its derivatives. The modular design allows for variation in key properties such as DNA specificity, transcriptional potency, and drug dependency. Production of GFP controlled cell-specific gene expression and facilitated functional perturbations in the mouse retina and brain. Further, retrofitting existing transgenic GFP mouse and zebrafish lines for GFP-dependent transcription enabled applications such as optogenetic probing of neural circuits. This work establishes GFP as a multifunctional scaffold and opens the door to selective manipulation of diverse GFP-labeled cells across transgenic lines. This approach may also be extended to exploit other intracellular products as cell-specific scaffolds in multicellular organisms.
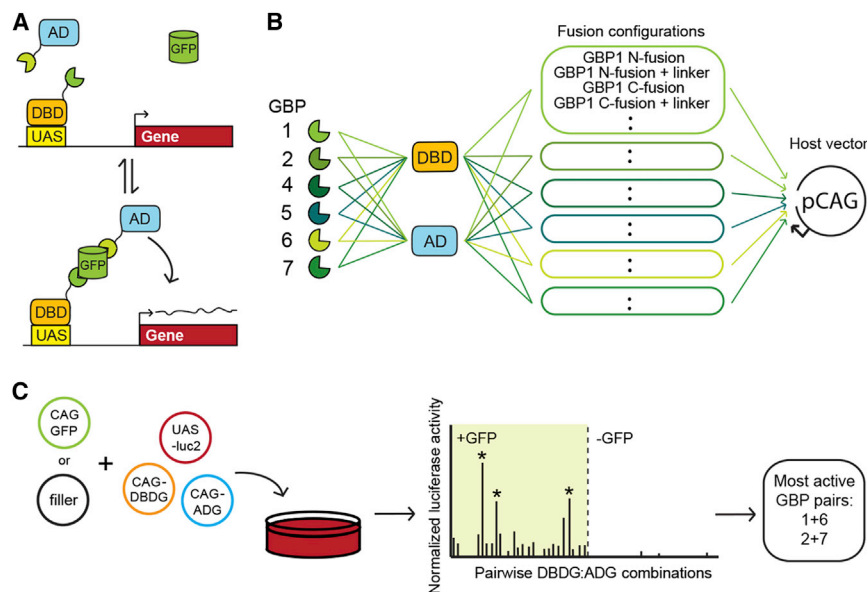
INTRODUCTION

Studies of multicellular organisms would be greatly facilitated by the ability to manipulate the activities of any genes within specific tissues or cell types. This is challenging to achieve in tissues with diverse cell types, such as the nervous system (Masland, 2004). To label and provide genetic access to diverse cell types, much effort has been devoted to generating transgenic organisms in which transgenes are placed under the control of large genomic fragments or endogenous gene loci. Transgenic lines expressing driver genes such as transcription factors or site-specific recombinases in specific cell populations can then be used to control the expression of genes in responder cassettes. However, the utility of individual lines is limited by a transgene's functional abilities; reporter lines expressing fluorescent proteins and histochemical enzymes are useful for labeling cells but cannot currently be used to control biological activities. To replace transgenes driven by the same *cis*-regulatory elements requires generation of additional transgenic lines. Such a procedure can be costly and lengthy for organisms such as the mouse. Thus, a key to conducting efficient and wide-ranging studies on existing and future model organisms is to increase the versatility of transgenic resources.

Owing to their ease of detection, GFP and its derivatives (Tsien, 1998) have become common markers of gene expression (Chalfie et al., 1994) across model organisms. Notably, thousands of transgenic GFP lines have been generated for the mouse (Gong et al., 2003). This growing and important resource reveals the expression pattern of many genes and provides strains in which GFP selectively labels many cell types of interest (http://www.gensat.org/index.html; Siegert et al., 2009). Transgenic GFP lines have enabled applications such as cell-type-specific transcriptome profiling, as well as targeted anatomical and physiological analysis (Huang et al., 2003; Siegert et al., 2012). However, functional manipulation of GFP-labeled cell types often requires the use of driver lines such as those that express Cre, which currently exist in limited numbers.

A system converting GFP expression into desired molecular outputs would enable existing and future transgenic GFP lines to be used directly for gene manipulation in specific cell types. Synthetic RNA devices have been engineered to convert the presence of an intracellular protein into gene expression output (Culler et al., 2010). Although promising, protein-responsive RNA devices await application in animals (Chang et al., 2012). Meanwhile, artificially derived binding proteins, herein including antibodies and unrelated proteins with ideal structures for evolving target recognition (Wurch et al., 2012), are being used

allowed for straightforward and predictable changes to critical features such as DNA binding specificity, transcriptional potency, and drug dependency. Our work extends the functionality of GFP into the regulatory realm, thus opening the door to selective manipulation of GFP-labeled cells across transgenic GFP lines and establishing components for the design of synthetic circuits.

RESULTS

Design and Isolation of GFP-Dependent Transcription Factors

In order to use GFP as a dimerizer, one has to identify GBP pairs that can bind to GFP at the same time. Suitable GBP pairs could then bring together fusion protein partners on the GFP scaffold. We obtained six GBPs for this purpose (Kirchhofer et al., 2010). Several GBPs were reported to bind additively to a preformed GFP-GBP1 complex when tested as purified proteins in vitro (Kirchhofer et al., 2010). However, it was unclear whether any of the identified pairs could co-occupy GFP, tolerate the addition of fusion partners, and induce the formation of biologically active complexes in cells. Furthermore, many possible GBP pair-wise combinations had not been tested for their ability to co-occupy GFP. To address these issues, we performed an in vitro reporter screen for GBP pairs that could induce the formation of an active transcription factor (Figure 1 and Extended Experimental Procedures). The Gal4 DNA-binding domain (DBD) and VP16 activation domain (AD) (Sadowski et al., 1988) were separately fused to GBPs in various configurations and placed under control of the ubiquitous CMV early enhancer/chicken β actin (CAG) promoter (Niwa et al., 1991) (Figure 1B). DBD-GBP (DBDG) and AD-GBP (ADG) fusion constructs were screened in pair-wise combinations for GFP-dependent activation of an upstream activating sequence-regulated luciferase (UAS-luc2) reporter in 293T cells. Functional DBDG/ADG pairs will be referred to as transcription devices dependent on GFP (T-DDOG). T-DDOGs employing GBP1+6 or GBP2+7 consistently gave the strongest reporter induction (Figures 1C, 2, and S1 available online) and became the focus of this study. To specify DBDG+ADG combinations, the DBD-GBPX fusion is listed in regular font, along with

intracellularly to target proteins in cells and organisms. Thus far, these reagents are used for target-centric purposes such as protein interference (Jobling et al., 2003), degradation (Caussinus et al., 2012), and modulation (Kirchhofer et al., 2010). Artificially derived binding proteins could possibly be a powerful platform to co-opt intracellular proteins as cell-specific signals that control synthetic circuits, without modifications to the target protein or reliance on the target protein's natural interactions or functions.

We explored whether artificially derived binding proteins can confer GFP with the ability to regulate genes. GFP seems relatively inert in many heterologous systems; it is freely diffusible in the cytoplasm, can enter the nucleus, confers low cytotoxicity, and has few interactions with host proteins (Trinkle-Mulcahy et al., 2008). The development of GFP-binding proteins (GBPs) from Camelid antibodies (Kirchhofer et al., 2010) has made possible the construction of GBP-fusion proteins noncovalently linking GFP to a variety of proteins in living cells (Caussinus et al., 2012). These reagents, termed nanobodies, are single-chain antigen-binding domains that are relatively small in size (~300–400 bp) and can be easily expressed in living cells (Rothbauer et al., 2006). Given the availability of multiple GBPs, we reasoned that GFP might be used as a scaffold to organize the formation of biologically active complexes. In one scheme, GFP would act like a small-molecule "dimerizer," bridging the association of distinct modular domains or protein fragments to reconstitute useful activities such as transcription and recombination (Jullien et al., 2003; Pollock and Clackson, 2002).
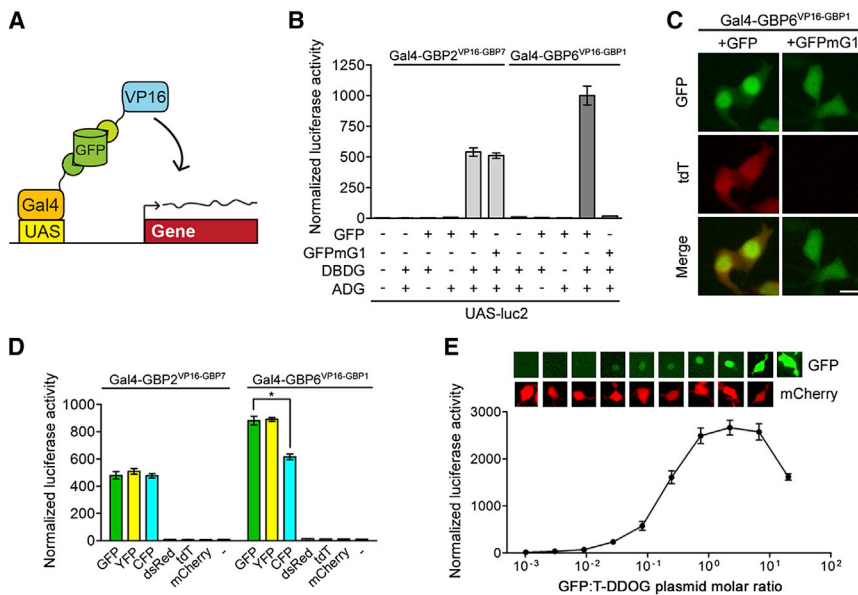
Here, we identified pairs of GBPs that can recruit tethered proteins onto the GFP scaffold, providing the means by which GFP-inducible systems can be built. We developed a GFP-dependent transcription system with these reagents, enabling control of any target gene for functional studies across tissues and organisms. The modular design of the transcription system

Figure 2. Characterization of the GFP-Dependent Transcription System

(A) Schematic of Gal4-based T-DDOGs.

(B) GFP-dependent activation of UAS-luc2 by Gal4-GBP6$^{VP16-GBP1}$ and Gal4-GBP2$^{VP16-GBP7}$. n = 9.

(C) Gal4-GBP6$^{VP16-GBP1}$ strongly activated UAS-tdT in the presence of GFP. Mutation of GBP1-binding residues in GFP (GFPmG1) abolished tdT activity. Scale bar, 10 μm.

(D) Specificity of T-DDOGs for different fluorescent proteins. n = 9; *p < 0.001.

(E) Activity of Gal4-GBP6$^{VP16-GBP1}$ in response to a varying amount of transfected GFP plasmids. The transfected DNA amount was kept constant among conditions, with CAG-mCherry (bottom) acting as a filler plasmid to compensate for reduction in GFP (top) plasmids. Panels show representative GFP and mCherry fluorescence in single cells for each corresponding data point below. n = 6. Plots are mean ± SD.

See also Figures S1 and S2 and Table S2.

the AD-GBPX fusion in superscript, giving DBD-GBPX$^{AD-GBPY}$. Specific T-DDOG configurations are tabulated in Table S1.

Characterization of the GFP-Dependent Transcription System In Vitro

The induced transcription output in 293T cells was found to be dependent on all components of the system, as removal of GFP, DBDG, or ADG from the transfection mixture resulted in loss of reporter activity (Figures 2B and S1). Reporter induction was further dependent on the ability of GBP to bind to GFP. Based on the GBP1 + GFP crystal structure (Kirchhofer et al., 2010), we mutated GFP residues expected to directly interact with GBP1. One such variant, GFPmG1, carries the mutations E143K and N147Q. Like GFP, GFPmG1 was localized to the nucleus by the VP16AD-GBP7 fusion protein (Figure S2). However, unlike GFP, GFPmG1 was not localized to the nucleus by the VP16AD-GBP1 fusion protein (Figure S2). In agreement with this, GFPmG1 induced strong UAS-reporter in the presence of Gal4-GBP2$^{VP16-GBP7}$, but not Gal4-GBP6$^{VP16-GBP1}$ (Figures 2B, 2C, and S1 and Table S2). These data confirm a requirement for GFP-GBP interactions and suggest that GBP2 and GBP7 do not depend critically on residue 143 or 147 for binding to GFP.

We also tested whether T-DDOG activity can be controlled by the GFP derivatives cyano and yellow fluorescent proteins (CFP and YFP) and the Discosoma-derived red fluorescent proteins dsRed, mCherry, and tdTomato (tdT) (Shaner et al., 2005). CFP and YFP induced Gal4-GBP2$^{VP16-GBP7}$ activity to a similar extent as GFP (Figure 2D). However, CFP had reduced ability to activate Gal4-GBP6$^{VP16-GBP1}$. This was expected because CFP differs from GFP at the GBP1-interacting residue 147 (Rothbauer et al., 2008). Also as expected, none of the red fluorescent proteins could induce T-DDOG activity. In support of this, red fluorescent proteins were diffusely distributed in the cell even when T-DDOG components were clearly localizing GFP to the nucleus (Figures 2C and 2E).

To evaluate the effect of GFP level on T-DDOG activity, we varied the amount of GFP plasmid delivered to 293T cells and examined UAS-luc2 expression in the presence of Gal4-GBP6$^{VP16-GBP1}$. The observations were consistent with those reported for small-molecule dimerizers (Ho et al., 1996). T-DDOG activity increased linearly with the amount of transfected GFP until a certain point, beyond which further increases in GFP led to reduction of activity. The reduced activity is likely due to titration of T-DDOG components by GFP. Interestingly, GFP was highly enriched in the nucleus at levels correlating with the rising phase of the dosage curve but spread into the cytoplasm at levels correlated with the declining phase of the curve (Figure 2E).

Modularity of GFP-Dependent Transcription System Permits Various Adjustments and Fine-Tuning

Transcription factors are highly modular (Luan et al., 2006; Sadowski et al., 1988). To exploit this feature for creating a diversity of T-DDOGs with varying properties, we substituted the transcription domains in our original GBP fusion library with other commonly used ones and conducted additional in vitro screens. Indeed, we were able expand and diversify the functional repertoire of T-DDOGs. T-DDOGs using the rTetR and LexA DBDs activated reporters bearing their respective binding sequences, tetO (included in tetracycline response element, TRE) and lexAop, only when GFP was present (Figures 3A–3E) (Butala et al., 2009; Schönig et al., 2010). The activities of rTetR-based T-DDOGs were further found to depend on doxycycline levels (Figure 3D). This drug dependency provides temporal control for the system.

T-DDOGs can also be adjusted to alter their transcriptional potency. The critical region for VP16AD function lies within a 12 amino acid peptide (VPmin) (Baron et al., 1997). We could predictably adjust the transcriptional activity of Gal4-GBP1$^{AD-GBP6}$ by either varying the number of VPmin repeats or the number
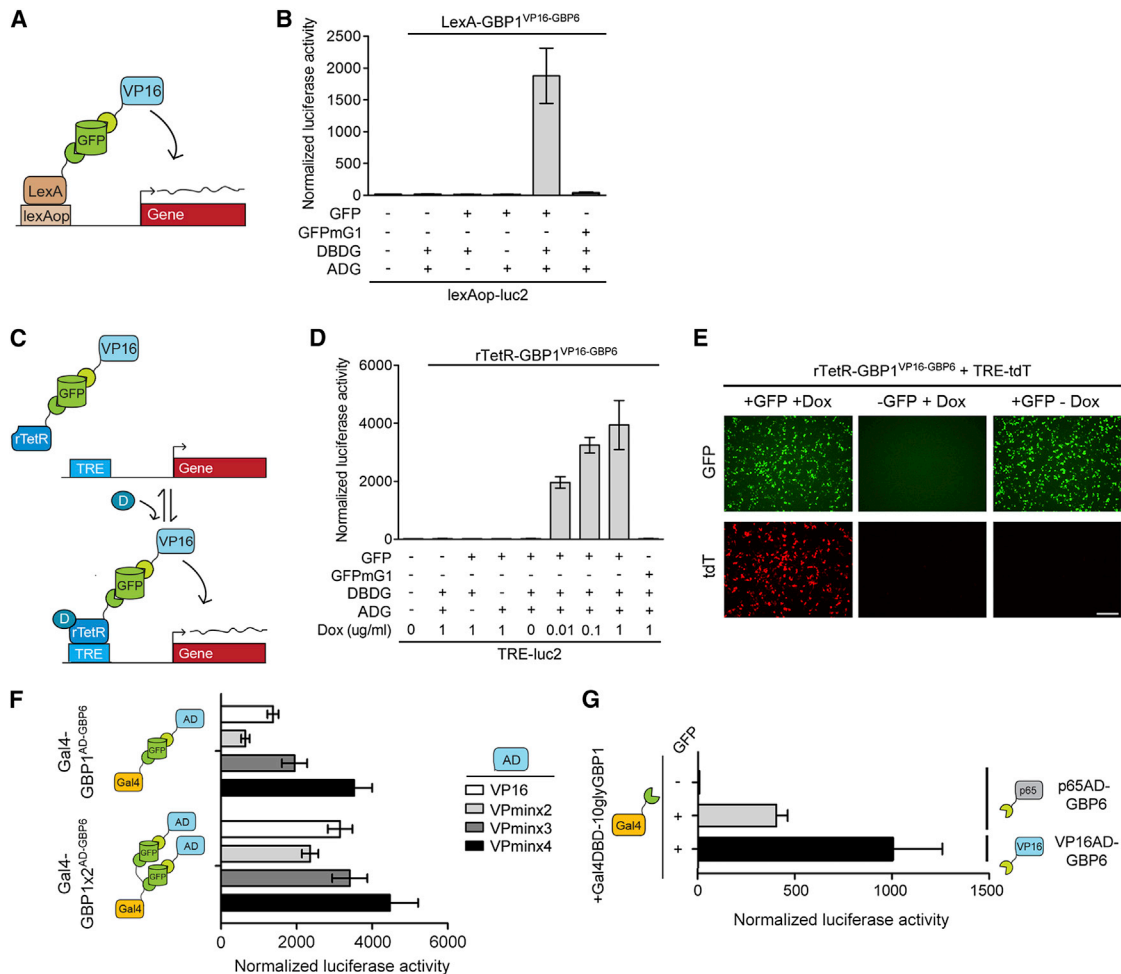
Figure 3. T-DDOGs Are Highly Adjustable

(A–E) T-DDOGs based on LexA (A) and rTetR (C) DBDs. Doxycycline is "D" in (C). TRE includes seven tetO sequences (C). (B) LexA-GBP1$^{VP16-GBP6}$ activated a lexAop-luc2 reporter only in the presence of GFP. n = 9. (D) rTetR-GBP1$^{VP16-GBP6}$ activated TRE-luc2 in a GFP- and doxycycline-dependent manner. n = 6–9. (E) Similar results were seen with TRE-tdT. Doxycycline was used at 1 μg/ml. Images were taken 16 hr posttransfection.

(F and G) Tuning T-DDOGs with adjustable DBDs and ADs. (F) Increasing the number of GBP1 on Gal4DBD (n = 6–9) enhanced the transcriptional potency for each ADG (n = 9). (G) Potency of p65AD compared to VP16AD. T-DDOGs used are Gal4-GBP1$^{p65-GBP6}$ and Gal4-GBP1-B$^{VP16-GBP6}$. n = 9. Scale bar, 100 μm. Plots are mean ± SD.

of GBPs fused to the DBD (Figure 3F). We further isolated potent T-DDOGs bearing the p65AD (Schmitz and Baeuerle, 1991), an alternative to VP16AD in synthetic transcription systems (Rivera, 1998) (Figure 3G). Overall, we consistently isolated potent T-DDOG variants using the GBP1+6 and GBP2+7 combinations, suggesting that these pairs can effectively recruit various combinations of fusion partners onto the GFP scaffold.

The GFP-Dependent Transcription System Can Be Used in the Mouse for Cell-Specific Gene Regulation

To evaluate whether GFP can control the activity of T-DDOGs in vivo, we used electroporation to introduce GFP, T-DDOGs, and UAS-tdT into the murine retina. In our initial tests, we found that overexpression of VP16AD caused mispositioning of rod photoreceptors in the outer nuclear layer (ONL), likely due to squelching of transcription machinery (Figure S3) (Gill and

Ptashne, 1988). To address this, we screened T-DDOGs with alternative ADs described above for their effects in the retina (Extended Experimental Procedures). We found that T-DDOGs made with VPminx2 and p65 ADs induced little to no disruption of normal rod positioning in the ONL (Figure S3). T-DDOGs bearing p65ADs were used in all subsequent experiments.

We examined how T-DDOG activity would respond to changes in GFP expression in the retina (Figures 4, S4, and S5 and Tables S3, S4, S5, and S6). When GFP was expressed under the broadly active CAG promoter, UAS-tdT was induced in GFP-expressing cell types of both the ONL and inner nuclear layer (INL) (Figure 4C). In contrast, little to no tdT signal was detected in electroporated retinas when GFP was excluded (Figures 4C and S4B). When the GFP expression pattern was manipulated with promoters specifically active in rods (Rho-GFP) (Matsuda and Cepko, 2004) or in ON bipolar cells (mGluR6-GFP) (Kim
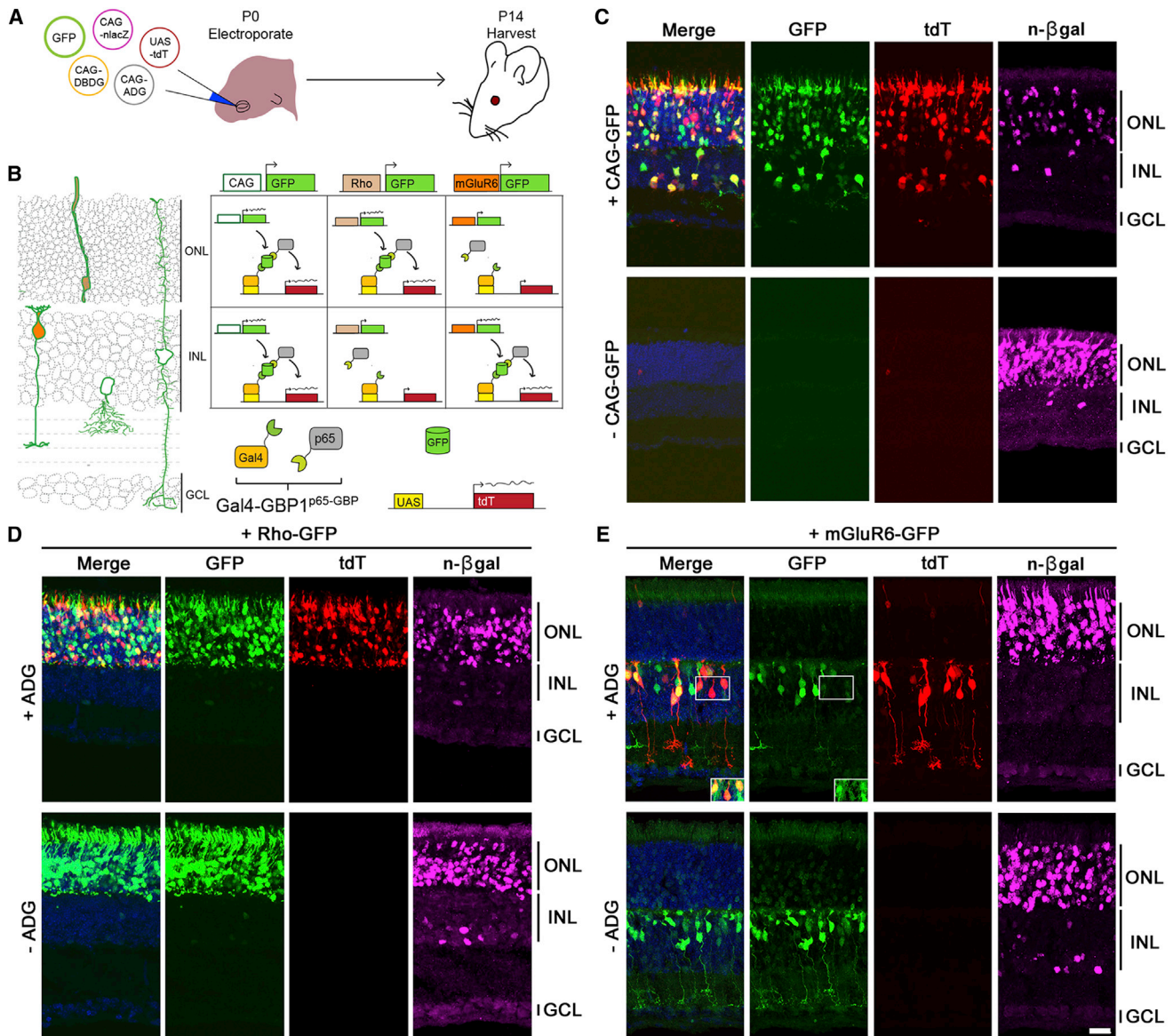
Figure 4. GFP Controls the Spatial Expression of Genes In Vivo

(A) Schematic of experiment.

(B and C) (B) (Left) In electroporated retinas, CAG-GFP expresses in multiple cell types (green outline). Rho-GFP expresses in photoreceptors of the ONL (beige fill). mGluR6-GFP expresses in ON bipolar cells of the INL (orange fill). GCL, ganglion cell layer. (Right) Anticipated UAS-tdT expression pattern aligned to left diagram (C) Gal4-GBP1$^{p65-GBP6}$ induces UAS-tdT only in the presence of GFP. n-βgal (magenta) is an electroporation marker.

(D and E) (Top) Rho-GFP and mGluR6-GFP induce tdT expression in rods and ON bipolar cells, respectively. (Bottom) tdT activation depends on ADG. Inset of (E) shows GFP and tdT colocalization upon GFP intensity enhancement. Merge panels includes GFP, tdT, and DAPI channels. Scale bar, 20 μm.

See also Figures S3, S4, and S5 and Tables S3, S4, S5, and S6.

et al., 2008), the tdT expression pattern shifted accordingly and was highly restricted to GFP-expressing cells (Figures 4D, 4E, S4, and S5). Cells labeled by the electroporation marker, nuclear β-galactosidase (n-βgal), but not GFP, did not express tdT (Figures 4C–4E). The efficiency of UAS-tdT activation, adjusting for the probability of a cell receiving all four necessary components for tdT activation, was ~56%–93%. Despite the lack of GFP signal amplification with antibodies, more than 90% to 95% of tdT-positive cells were positive for GFP expression in all cases (Table S6). Unexpectedly, we detected faint mGluR6-GFP expression in the ONL, which was not seen without the introduction of T-DDOGs. This low level of GFP induced little to no tdT expression (Figures 4E and S4G). Follow-up experiments indicated that Gal4-GBP1$^{p65-GBP6}$, but not Gal4-GBP2$^{p65-GBP7}$, stabilized a low level of ONL GFP leaking from the mGluR6 promoter (see Supplemental Information). This suggests that
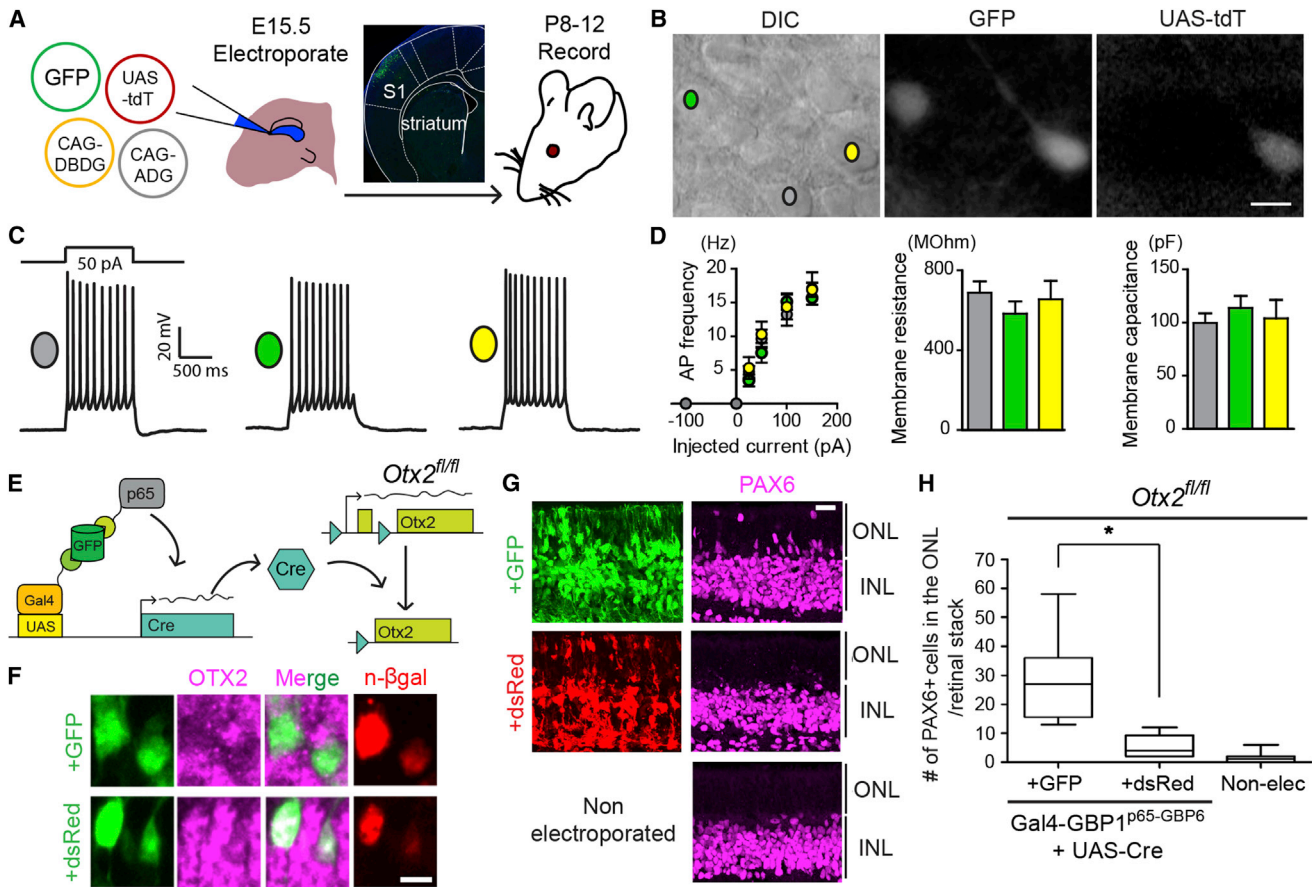
Figure 5. T-DDOGs Support Electrophysiological and Gene Perturbation Studies in the Central Nervous System

(A) Electroporation setup for neuronal recordings. Micrograph shows GFP in electroporated primary somatosensory cortex (S1).

(B–D) (B) Image of an acute brain slice from an electroporated mouse. Scale bar, 10 μm. Three categories of pyramidal layer 2/3 S1 neurons were recorded from brain slices: nonfluorescent controls (gray), GFP+ (green), and GFP+/tdT+ (yellow). (C) Representative single current-clamp trace of action potentials in response to a 50 pA, 1,000-ms-long step current injection. (D) Plots show action potential (AP) frequency upon current injection, as well as input resistance and membrane capacitance of recorded cell classes. p > 0.5 for all comparisons (n = 8–10 neurons per condition). Plots show mean ± SEM.

(E–H) GFP-dependent excision of $Otx2^{fl/fl}$ in the retina. (E) P0, $Otx2^{fl/fl}$ mouse retina was electroporated with T-DDOG components and UAS-Cre and either CAG-GFP or CAG-dsRed. (F–H) (F) Loss of OTX2 was confirmed by OTX2 immunostaining and (G and H) ectopic PAX6+ ONL cells. n = 10 stacks, 5 retinas per electroporated condition. For nonelectroporated retina, n = 19 stacks, 10 retinas. Boxplots show median, maximum, and minimum values. Retinal stacks are 12-μm-thick confocal images. *p < 0.001. n-βgal marks electroporated cells in (F). Scale bar, 5 μm in (F) and 20 μm in (G and H).

See also Figures S6 and S7 and Table S7.

Gal4-GBP1$^{p65-GBP6}$ can reveal GFP expression that is normally below the threshold of detection, whereas Gal4-GBP2$^{p65-GBP7}$ allows for gene manipulation without revealing subdetection levels of the native GFP expression pattern. Overall, these results showed that GFP could be used as a cell-specific regulator of T-DDOG activities in the mouse.

Utility of GFP-Dependent Transcription System for Electrophysiological Studies and Gene Perturbations

To evaluate whether T-DDOGs altered the properties of neurons, we electroporated GFP, T-DDOGs, and UAS-tdT into the somatosensory cortex and examined various properties of cortical neurons from ~1.5-week-old mouse brains. We compared pyramidal neurons expressing the full set of T-DDOGs, showing both GFP and tdT, with those that expressed GFP alone, as well as with neighboring neurons that lacked fluo-

rescence (Figures 5A–5D). TdT signal was not observed in GFP-negative neurons in the acute slices (data not shown). We found that excitability and passive membrane properties were similar for the three groups of neurons (Figures 5C–5D and Table S7) and were consistent with intrinsic cellular properties previously reported for cortical neurons of this age (Oswald and Reyes, 2008). Moreover, transducing T-DDOGs did not impact morphological features such as dendritic spine density and length (Figure S6 and Table S7). Thus, T-DDOGs are compatible with electrophysiological assays and do not induce functional and structural alterations in the developing brain, within the tested time frame.

We further evaluated the utility of T-DDOGs for deriving biological effects in developing tissues. *Otx2* is a homeobox gene that is necessary for photoreceptor specification in the retina (Nishida et al., 2003). We used GFP to induce Cre-mediated
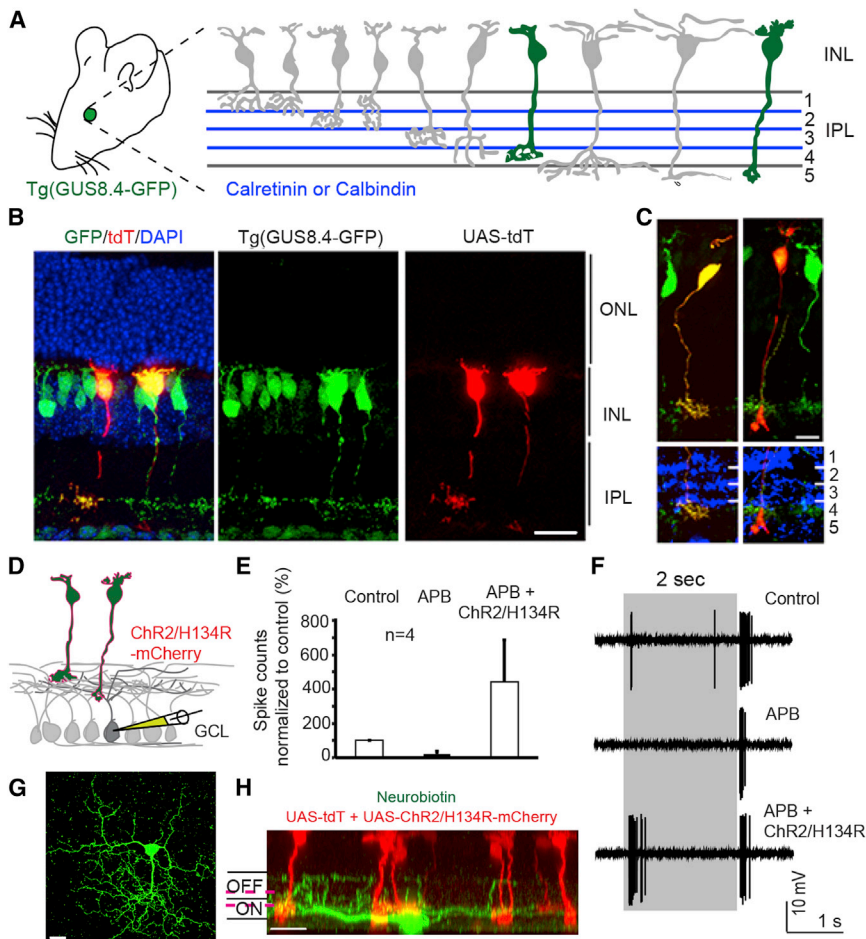
(A) Tg(GUS8.4GFP) expresses GFP in type 7 cone bipolar and rod bipolar cell types (green fill) of the retina. Adopted schematic (Ghosh et al., 2004).

(B) Cryosection of electroporated Tg(GUS8.4GFP) retina expressing Gal4-GBP2^p65-GBP7 and UAS-tdT. Scale bar, 20 μm.

(C) Type 7 (left) and rod bipolar (right) cell types labeled by UAS-tdT. Anti-Calretinin (left) or anti-Calbindin (right) staining identify specific layers of the IPL. Scale bar, 10 μm. GFP was immunostained in (B and C).

(D) Schematic of ChR2 experiment. Electroporated Tg(GUS8.4-GFP) retinas expressing 10× UAS-ChR2/H134R-mCherry and 5×UAS-tdT were analyzed for ChR2-mediated responses in random GCL cells.

(E) Cumulative plot of ON responses in GCL cells. Number of spikes counted during the first 300 ms after stimulus onset, normalized to control (minus APB). APB blocks ON responses originating from photoreceptors. Plots are mean ± SEM (n = 4 per condition).

(F) Spiking response of a GCL cell. Gray bar, duration of light stimulus. Response to normal light stimuli under control condition (top) or in the presence of APB (middle). Light stimuli focused on INL activate ChR2/H134R in the presence of APB (lower).

(G and H) Top and side views of a neurobiotin-filled (green) ganglion cell identified by light stimulation of ChR2. Magenta lines indicate level of anti-Chat bands (not shown). Scale bar, 20 μm.

excision of a floxed *Otx2* allele (*Otx2^fl/fl*) (Tian et al., 2002) in mouse retinas ex vivo, with Cre being under the regulation of the UAS promoter (Figures 5E–5H). This led to the loss of OTX2 protein and the expected ectopic gain of PAX6 in the ONL (Nishida et al., 2003) (Figures 5F and 5G). Conversely, OTX2 levels were not significantly perturbed when GFP was replaced in the same experiment with dsRed (Figure S7). A slight increase in PAX6+ ONL cells above background values was likely due to leakage of UAS-Cre under experimental conditions. Thus, T-DDOGs will be useful for converting GFP expression into desired Cre-mediated genetic changes using a variety of existing conditional alleles. Taken together, these results showed that T-DDOGs are suitable for gene perturbations in the mouse and are compatible with assays of cellular function.

Retrofitting Transgenic GFP Lines for GFP-Dependent Manipulation of Genes and Neural Circuits

We examined whether T-DDOGs can retrofit transgenic GFP lines for cell-specific gene manipulations. In the mouse retina, visual information detected by rod and cone photoreceptors is transmitted to bipolar cells and ultimately to ganglion cells. Among bipolar cells, the rod bipolar cell type receives input from rods, whereas many types of cone bipolar cells receive

input primarily from cones. Currently, almost none of the cone bipolar types can be singly isolated for genetic manipulation, but multiple GFP lines do label subsets of bipolar types (Siegert et al., 2009; Wässle et al., 2009). The α-gustducin-GFP transgenic line, Tg(GUS8.4-GFP) (Huang et al., 2003), expresses GFP in type 7 cone bipolar cells and in rod bipolar cells (Figure 6A). Both cell types respond to light increments and are called ON bipolar cells. Introduction of T-DDOGs and UAS-tdT into Tg(GUS8.4-GFP) retinas resulted in tdT induction selectively in these two cell types; identification was based on morphology and axonal stratification in the inner plexiform layer (IPL), aligned to the IPL markers Calbindin or Calretinin (Ghosh et al., 2004) (Figures 6A–6C). Importantly, 98.9% of tdT+ cells were positive for GFP expression (n = 91 cells, sampled from three retinas).

One exciting use of T-DDOGs would be to express light-sensing ion channels in cell types labeled by transgenic GFP for refined, optogenetic probing of neural circuits (Yizhar et al., 2011). We explored this possibility by expressing a UAS-regulated channelrhodopsin-2 (ChR2) variant, H134R (Nagel et al., 2005) in Tg(GUS8.4-GFP)-labeled cells. We asked whether light-driven ChR2 activation in GFP-labeled bipolar cells could trigger downstream spiking responses in cells of the ganglion cell layer (GCL) (Figure 6D). Electroporated retinas were
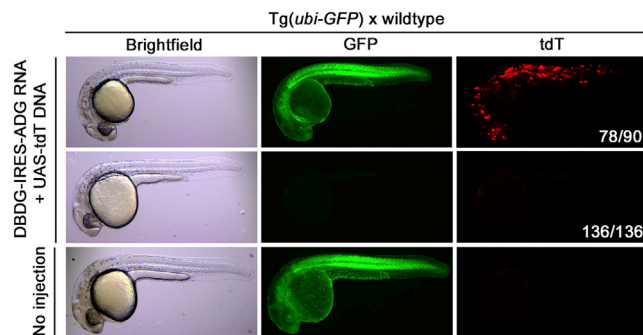
Figure 7. GFP-Dependent Transcription in Transgenic Zebrafish
Embryos from Tg(*ubi-GFP*) × wild-type outcrosses were microinjected with DBDG-IRES-ADG (Gal4-GBP1[p65-GBP6]) RNA and UAS-tdT DNA at the one- to two-cell stage and examined 1 to 2 days postfertilization. Images represent X number of embryos out of Y number of injected embryos (X/Y), shown in white font in tdT panel.

presented with two different light stimuli, and recordings were performed on GCL cells. The first stimulus had low light intensity and could evoke photoreceptor-mediated responses in GCL cells but was not bright enough to activate ChR2. We used this stimulus to select GCL cells that responded to both light increments and decrements (ON/OFF cells) (Figures 6E and 6F). We next blocked synaptic communication between photoreceptors and ON bipolar cells with 2-amino-4-phosphonobutyrate (APB) (Slaughter and Miller, 1981) and presented the retina with a brighter light stimulus that could activate ChR2. Because ON/OFF GCL cells receive excitatory input from ON bipolar cells, some of these cells should be connected via excitatory synapses (directly or indirectly) to ChR2-expressing ON bipolar cells. Indeed, the brighter stimulus elicited ON responses in some recorded GCL cells in the presence of APB (Figures 6G and 6H). In contrast, recordings made from ON and ON/OFF GCL cells in nonelectroporated regions of multiple retinas did not reveal any response after the onset of the brighter stimulus in the presence of APB (data not shown). Thus, ChR2 activation in rod bipolar or ON cone bipolar cells was robust enough to evoke neurotransmitter release from bipolar cells. Further, the resulting current in GCL cells was large enough to reach spike threshold and evoke spiking responses. These results showed that T-DDOGs could turn on optogenetic tools in transgenic GFP cells, permitting functional interrogation of neural circuits.

Utility of T-DDOGs in Zebrafish

In order to determine whether T-DDOGs can direct GFP-dependent activities in other organisms, zebrafish were tested. Here, T-DDOG components were translated from one bicistronic transcript by linking DBDG and ADG components with an internal ribosome entry site (IRES) element. We microinjected RNAs with this structure into ubiquitin-GFP transgenic (Tg(*ubi-GFP*) (Mosimann et al., 2011) zebrafish embryos in a transient reporter assay. Indeed, mosaic UAS-tdT expression was clearly induced in 78 of 90 injected GFP+ embryos, but not in the 136 injected GFP− embryos (Figure 7). This demonstrates the utility of this system across species.

DISCUSSION

Fluorescent proteins are useful for illuminating cells and cellular processes. Moreover, their apparent lack of connection to many host protein networks makes them ideal scaffolds upon which one can build synthetic complexes with desirable biological activities. We demonstrated this principle here by using GFP to induce formation of a hybrid transcription factor for gene regulation purposes. The ability to use GFP for gene regulation now enables one to experiment with many GFP-labeled cell types without the need to create new cell-specific driver lines or to discover new cell-specific promoters. This system can be used for gene overexpression and gene deletion (Figures 4, 5, 6, and 7) and should be able to perform RNA interference (RNAi) knockdown (Dickins et al., 2007; Dietzl et al., 2007). Activities of the system can be controlled by GFP and its derivatives, but not by red fluorescent proteins, thereby allowing the two types of fluorescent proteins to be used independently in the same experiment. Red fluorescent proteins can likely be used as scaffolds as well. In particular, monomeric variants such as mCherry would be straightforward to use, as they do not undergo obligate dimerization or tetramerization (Campbell et al., 2002).

Selective Control of GFP-Labeled Cells in Transgenic GFP Organisms

The development and functions of complex multicellular organisms depend upon the activities of a large number of distinct cell types. To investigate these activities in the nervous system, for example, many molecular tools have been developed for anatomical circuit tracing, as well as physiological control (Wickersham and Feinberg, 2012; Yizhar et al., 2011). However, the full potential of these tools can only be realized when one can selectively control them in any cell type in the nervous system. We demonstrated that the diverse transgenic GFP lines available in the mouse and other organisms would be useful for cell-specific manipulation of genes and neural circuits. In the mouse, such manipulations are performed primarily with recombinases such as Cre. Although we anticipate that the number of cell-specific Cre mouse lines will continually increase along with that of GFP lines, each collection of lines will be independently useful for some applications and will be complementary for other applications. As discussed below, the use of GFP is not necessarily limited to transcriptional control. Nevertheless, gene regulatory systems based on transcription factors differ fundamentally from those based on recombination strategies. As recombination induces alterations to DNA sequences, this typically results in permanent changes in gene expression. In contrast, transcription systems are reversible. Recombination systems are especially useful for targeting cells with a common gene expression history and for long-term transgene expression independent of initial induction signals. However, the irreversibility of recombination events can result in the manipulation of undesired cell types. This should be less of a problem for a transcription system, as continual expression of GFP, in our case, is required to maintain the transcription of target genes. Although GFP may persist for a prolonged period of time after its own transcription has been shut off, this effect may be advantageous in certain applications, such as when it is desirable to achieve a

moderately prolonged but reversible gene expression effect. When temporal control of gene expression is desired regardless of GFP expression, rTetR-based T-DDOGs should be useful as they are additionally controlled by drug treatment (Figures 3C–3E) and could take advantage of the various TRE reagents available (Schönig et al., 2010).

There are additional reasons to use GFP lines for cell-specific targeting. First, not all definable cell types can be specifically targeted by a single driver line (Dymecki et al., 2010). Restriction of target gene expression may be accomplished by intersecting GFP expression with expression of T-DDOG components, other transcription systems, and/or recombination systems. Second, position effects can sometimes unexpectedly activate GFP in unique cell types. For example, this is thought to be the case for the Tg(GUS8.4-GFP) line and the Tg(Thy1-XFP) collections (Huang et al., 2003; Feng et al., 2000). Replacement of transgenes typically requires the generation of new transgenic lines and can result in changes to cell specificity of transgene expression. Emerging site-specific, genome-editing strategies hold promise for enabling efficient swapping of transgenes while minimizing changes to cell specificity (Cong et al., 2013; Gohl et al., 2011; Mali et al., 2013). However, it still takes a relatively long time, as well as significant expense, to generate, characterize, and maintain modified transgenic mouse lines.

The GFP-dependent transcription system should find applications beyond mice and zebrafish. As T-DDOGs are built from protein parts commonly used in other model organisms such as *Drosophila*, other communities can easily adopt T-DDOG components for use in concert with existing GFP driver and responder lines, as well as transient gene delivery vectors. In addition, the modularity of this system allows for a seemingly unlimited number of T-DDOGs to be created according to user demands. Notably, T-DDOGs with customizable DNA-binding specificity (Hsu and Zhang, 2012) would allow for targeted control of endogenous loci without the need for responder cassettes.

Practical Considerations for T-DDOG Use

Although T-DDOG activities are highly dependent on GFP expression, whether one succeeds in converting an observed GFP expression pattern into corresponding gene output pattern depends on several factors. First, cells expressing GFP at low levels, or transiently, may evade detection in the initial stages of characterization. Such "background" GFP expression may be detected by certain T-DDOG configurations. Differing fusion protein stability and/or differing GBP affinity for GFP probably contribute to differences in T-DDOG sensitivity. Specifically, we found that Gal4-GBP1$^{p65-GBP6}$ promoted detection of normally undetectable GFP expression from mGluR-GFP in photoreceptors. However, little to no T-DDOG-mediated expression occurred in these cells. As an mGluR6-driven Cre construct was found to induce recombination in photoreceptors, in addition to bipolar cells (data not shown), we interpret the photoreceptor GFP signal as reflecting leakage from the mGluR6 promoter, as well as stabilization of the leaky GFP by Gal4-GBP1$^{p65-GBP6}$. Because Gal4-GBP2$^{p65-GBP7}$ did not reveal the leaky mGluR6-GFP expression and its corresponding T-DDOG did not induce reporter output in photoreceptors,

Gal4-GBP2$^{p65-GBP7}$ may be used for cases when it is not desirable to reveal GFP expression normally below detection threshold. A second issue regards T-DDOG detection of transient GFP expression during early development. This could be addressed by using rTetR-based T-DDOGs for temporal control or by restricting T-DDOGs expression to late progenitors or post-mitotic cells, as is possible with electroporation, viral vectors, and/or late-expressing promoters.

Very high levels of GFP expression also require consideration, as too much GFP may saturate GBP binding sites, thereby preventing assembly of T-DDOGs (Figure 2E). Nonetheless, we could induce strong T-DDOG readout in Tg(CRX-GFP), a very strong GFP-expressing mouse line (Samson et al., 2009) (data not shown). When necessary, there are approaches to overcome the issue of excessive GFP expression. First, one can capture T-DDOG activity using a recombinase as the T-DDOG readout. We demonstrated that T-DDOGs could drive expression of Cre to induce irreversible gene expression changes regardless of changes in GFP level. Second, we showed that one could increase the number of GBPs on the DBDG component; this is expected to enhance the GFP binding capacity of the system. Lastly, higher levels of expression of T-DDOGs should be able to balance high GFP levels. Transgenic lines expressing T-DDOGs at high, medium, and low levels should be sufficient for a research community to manipulate a broad range of transgenic GFP lines.

Here, we demonstrated that electroporation or microinjection could immediately be used to deliver T-DDOGs to receptive tissues and organisms for manipulation of GFP-labeled cell types. Additionally, it should require little effort to extend the delivery route to viral vectors. The two components of T-DDOGs can be linked by IRES elements or 2a peptides for expression from a single promoter (Figure 7). Each component is relatively small, ranging from ~500 bp to ~1.2 kbp in length, allowing both T-DDOG components to fit into popular viral vectors such as adeno-associated viruses (AAVs) (Yizhar et al., 2011). Responder cassettes can also be delivered virally or by electroporation (Figures 4, 5, and 6). Transient delivery methods usually do not provide access to all possible cell types within a tissue or organism; this can be taken into account during experimental design. For example, any retinal cell class is accessible given the right choice of AAV serotypes or electroporation method (Matsuda and Cepko, 2004, 2007; Watanabe et al., 2013). Furthermore, the inherent cell-type specificity of certain gene delivery methods can be exploited to subtract undesired GFP-labeled cell types from being manipulated. For some applications, it will be desirable to deliver T-DDOGs to all cells in a tissue, or to entire organisms. Transgenic lines expressing T-DDOGs under the control of broadly active promoters would meet these needs, and these tools are under development.

Whenever exogenous components are delivered to cells, one should be cautious of unintended effects. Potential side effects from AD overexpression have long been recognized (Gill and Ptashne, 1988), but applications with transcription factors continue to grow (Schönig et al., 2010; Venken et al., 2011) and drive meaningful discoveries (Kinoshita et al., 2012; Miyamichi et al., 2011). As we found here, problems with an AD may be overcome by testing ADs differing in origin and transcriptional

potency. Alternatively, one can reduce expression of a given T-DDOG. IRES-linked cassettes typically give lower expression of genes in the second position relative to those in the first position (Mizuguchi et al., 2000). This could be used to express the DBDG component at high levels while keeping the ADG levels relatively low.

Fluorescent Proteins as Multifunctional Switches for Heterologous Systems

As GFP does not have any committed regulatory function in host cells, it may be co-opted for other regulatory purposes. To realize GFP's full potential, additional GFP-binding reagents and engineering efforts will be needed to expand its functionality and to improve the performance of GFP-dependent devices. Beyond transcription, the GFP scaffold should be able to regulate other activities such as recombination (Jullien et al., 2003) and proteolysis (Wehr et al., 2006). Although the GBP1 binding epitope on GFP has been revealed by X-ray crystallography (Kirchhofer et al., 2010), it is unclear how GBP2, 6, or 7 bind to GFP. Structural understanding of how GBP pairs co-occupy GFP would facilitate the design of other GFP-inducible complexes, such as when protein fragments have to be positioned in strict orientations.

Eventually, fluorescent proteins may become preferred transgenes for organisms with long generation times, such as rodents and primates. Because the expression pattern of fluorescent proteins can be characterized from the first set of transgene carriers, any experimental manipulation of labeled cells could be conducted within the same generation by transient device delivery or in the next generation by mating with transgenic device carriers. Also, as one can retroactively build systems to exploit fluorescent proteins for different purposes, it may become unnecessary to generate redundant lines driving different transgenes selectively in the same cell populations. Lastly, engineering of small-molecule ligands that regulate fluorescence would even enable one to use fluorescent proteins exclusively for gene control without interfering with the imaging of other spectrally overlapping probes (Kumagai et al., 2013).

Perspective on Targeting Intracellular Products for Cell-Specific Control

Many intracellular products, such as RNA and proteins, are expressed in a cell-specific manner and could potentially be exploited as spatial signals to control synthetic circuits in multicellular organisms. Here, we demonstrated that artificially derived binding proteins are useful for co-opting an intracellular protein, GFP, for this purpose. Because this approach does not require any modification of the target molecule or rely on the molecule's natural interactions or functions, it may be generalizable to any intracellular product for which artificially derived binding proteins can be selected. Certainly, GFP seems to be an ideal target because it is an exogenous molecule that shows little connection to host protein networks. However, other exogenous molecules, such as β-galactosidase or Cre recombinase, should also be useful as scaffold proteins. Furthermore, endogenous molecules probably exhibit a spectrum of connectivity within the host interactome, and a subset might be appropriate for conferring cell-specific manipulations in multicellular organisms.

The ability to use intracellular products simply as cell-specific scaffolds would enhance one's ability to target and control cells in nonmodel organisms where transgenic lines are not available.

EXPERIMENTAL PROCEDURES

Detailed methods, data analysis, and reagents used can be found in the Extended Experimental Procedures.

Animals

All animal experiments performed were approved by the Institutional Animal Care and Use Committee at Harvard University. Animal information is in the Extended Experimental Procedures.

Molecular Biology and Screens

Using standard techniques, coding sequences of GBPs were fused to those of DBDs or ADs in many configurations, and the products were inserted into pCAG (Niwa et al., 1991). Pairwise combination of DBDG and ADG constructs were then introduced into 293T cells or the mouse retina for screens. See the Extended Experimental Procedures for details.

In Vitro Luciferase Assays

Plasmids encoding CAG-driven GFP, DBDG, and ADG were transfected via polyethylenimine (PEI) into 293T cells along with plasmids encoding UAS-luc2 and Renilla luciferase or UAS-tdT. Cells were harvested 24 hr later for dual-luciferase assay (Promega) or imaged 16 hr later. All transfections except for the dosage curve were done at a 1:1:1 (GFP:DBDG:ADG) plasmid molar ratio.

In Vivo Electroporations

P0 CD1 retinas were microinjected with plasmids into the subretinal space and subjected to electroporation (Matsuda and Cepko, 2004). Plasmids encoded DBDG, ADG, CAG-nlacZ (expresses the electroporation marker n-βgal), UAS-tdT, and different promoter-GFP constructs. Electroporated retinas were harvested at P14, immunostained for n-βgal, and imaged by confocal microscopy. At P3–5, Tg(GUS8.4GFP) retinas were electroporated with plasmids encoding T-DDOG components, UAS-driven constructs, and CAG-nlacZ; retinas were harvested between 3 and 4 weeks of age for UAS-tdT detection and between 8 and 10 weeks of age for 10×UAS-ChR2/134R stimulation. Wherever applicable, retinas were immunostained with anti-GFP or anti-dsRed to visualize processes. Anti-Calbindin or anti-Calretinin label layers in the IPL.

Neuronal Recordings

C57BL/6 embryos were electroporated with plasmids encoding CAG-driven GFP, DBDG, ADG, and UAS-tdT into the lateral ventricle at embryonic day 15.5. Acute brain slices were prepared from electroporated 1- to 2-week-old mice using standard procedures. Whole-cell current clamp recordings were performed on GFP−, GFP+, and GFP+/tdT+ cortical layer 2/3 pyramidal neurons in regions of dense electroporation. For ChR2/H134R experiment, electroporated retinas from 8- to 10-week-old Tg(GUS8.4GFP) mice were flat mounted, and loose cell-attached patch clamp was performed on GCL cells that had mCherry/tdT+ bipolar cells in their dendritic fields. Photoreceptors were stimulated by light focused on the outer segments of photoreceptors at a light intensity of 1.3×10^3 R*/s. 20 μM APB was used whenever applicable. ChR2 was stimulated by light focused on the bipolar cell layer at ~10^8 R*/s for 2 s. Epifluorescence or two-photon microscopes were used to identify fluorescent cells.

Otx2 Removal Experiment

P0 *Otx2*$^{fl/fl}$ (Tian et al., 2002) retinas were electroporated ex vivo (Emerson and Cepko, 2011) with plasmids bearing Gal4-GBP1$^{p65-GBP6}$, UAS-Cre, and CAG-nlacZ along with either GFP or dsRed-expressing plasmids. Retinas were cultured ex vivo, harvested at P8, immunostained for OTX2, PAX6, and/or n-βgal expression, and subjected to confocal imaging.

Zebrafish Microinjections

DBDG and ADG coding sequences of Gal4-GBP1$^{p65-GBP6}$ were linked by an IRES element, subcloned into pCS2+, and transcribed in vitro from the SP6

promoter (mMessage mMachine SP6 RNA Kit, Ambion). RNAs were subjected to LiCl precipitation. One- to two-cell embryos were injected with 40 ng/μl RNA encoding IRES-linked T-DDOG and 25 ng/μl of NotI-linearized UAS-tdT DNA. GFP+ and GFP− embryos, obtained from outcrosses of heterozygote Tg(*ubi-GFP*) (Mosimann et al., 2011) males to wild-type Tubingen females, were blindly injected with the same RNA/DNA mixture in the same experiment such that the injection success rate for both genotypes should be similar. Injected embryos were incubated at 28°C, and survivors were analyzed for GFP and tdT expression 1 to 2 days postfertilization.

Statistical Analysis

Two-tailed Student's t test assuming unequal variance was used for all comparisons except cortical recording analysis, for which one-way ANOVA was used. $p > 0.05$ is judged as statistically significant.

See the Supplemental Information for detailed methods and reagents used.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures, seven figures, and seven tables and can be found with this article online at http://dx.doi.org/10.1016/j.cell.2013.07.021.

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